IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 7350**
Masahiko KURODA et al.	:	Attorney Docket No. 2006_0025A
Serial No. 10/564,481	:	Group Art Unit 1634
Filed March 7, 2006	:	Examiner Katherine D. Salmon
METHODS FOR DIAGNOSING ENDOMETRIOSIS-RELATED DISEASES	:	**Mail Stop: RCE**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents,
Washington, D.C.

Sir:

I, Masahiko Kuroda, the undersigned, a citizen of Japan, residing at 1-7-11 Minami-Ogikubo, Suginami-ku, Tokyo, do hereby declare:

1. That I am an inventor of the above-identified application.

2. That I graduated from The University of Tokyo on March 31, 1993 with a degree in M.D., Ph.D.

3. That I was Assistant Professor in The University of Tokyo (1993-1996), Assistant Professor, New York University (1996-1998), and I am now Associate Professor in Tokyo Medical University (1998-present).

4. That I have published papers as listed below.

(1) Oikawa, K, Kuroda, M et al.: Increased expression of IgE-dependent histamine-releasing factor in endometriotic implants. J Pathol, 199: 318-323, 2003.

(2) Oikawa, K, Kuroda, M et al.: Dioxin Stimulates Synthesis and Secretion of IgE-Dependent Histamine-Releasing Factor. Biochem Biophys Res Commun, 290: 984-987, 2002.

(3) Kuroda Met al.: Male Sterility and enhanced radiation sensitivity in TLS-/- mice. EMBO J, 19: 453-462, 2000.

(4) Kuroda M et al.: Induction of a novel secreted protein by the myxoid liposarcoma oncogene. Proc Natl Acad Sci USA, 96: 5025-5030, 1999.

5. That in order to show the enablement of claims 1, 6, 7 and 8 of the above-identified application, I have under my control and direction conducted the following experiments. The particulars and results of the experiments are set forth herein below.

Experiment

This experiment was done to measure expression levels of HRF gene in menstrual blood from normal subjects and endometriosis patients.

A. Procedures

cDNA was synthesized from menstrual blood (3 ml) of normal subject (n=7) and endometriosis patient (N-30), respectively, and real-time PCR was performed by using STRATAGENE Mx3005P™ Real-Time PCR System with STRATAGENE Brilliant® Multiplex QPCR Master Mix.

(i) Composition of PCR solution

2xMaster Mix		10 μl
β-actin primers	sense (40 μM)	0.042 μl
	antisense (40 μM)	0.042 μl
HRF primers sense (40 μM)		0.042 μl
	antisense (40 μM)	0.042 μl
β-actin probe (5 μM)		0.069 μl
HRF probe (5 μM)		0.069 μl
ROX reference dye (2 μM)		0.3 μl
H_2O		8.394 μl
Template 1st strand cDNA		1 μl

(ii) PCR primers and probes

β-actin primers:	5'-GCTGCCCTGAGGCACTCT-3'
	5'-CGGATGTCCACGTCACACTT-3'
HRF primers:	5'-TTCAGTCGCCATCATGATTATCTAC-3'
	5'-GCGATCTCCCGGATCTTG-3'

β-actin probe: 5' CF560-AGCCTTCCTTCCTGGGCATGGAGTC-BHQ1 3'

HRF probe: 5' FAM-CCTCATCAGCCACGATGAGATGTTCTCC-BHQ1 3'

(iii) PCR protocols

	Degree	Time	Acquisition
	95°C	10 min	off
50 cycles [	95°C	30 sec	off
[	60°C	1 min	on (at the end)

(iv) Measurement

Fluorescent data was adjusted with ROX data by using STRATAGENE MxPro™ Software. The relative expression level of each sample was measured from a calibration curve of dilution series, and was divided by β-actin data and standardized.

B. Results

Figure A (attached herewith) shows the results of real-time PCR. Expression levels of HRG gene in menstrual blood from endometriosis patients were much higher than that from normal subjects.

Statistical analysis of Figure A by the Declarant

1. **Fluorescent signal intensity of HRF cDNA in menstrual samples from endometriosis patents and normal subjects**

	Average	Unbiased Variance	Standard Deviation
Patients (N=30)	1.738	0.109361379	0.330698321
Normal subject(N=7)	0.285174286	0.015595238	0.124880896

2. **Student's t-test (positing equal variance)**

Difference of Average	Degree of Freedom	t-value	two-sided p-value	t(0.975)
1.452285714	35	11.32791182	2.944131E-13	2.030107915

Interval estimation for differences of population average:

Degree of reliability	95%
Lower limit	1.192017381
Upper limit	1.712554047

3. **Welch's t-test (not positing equal variance)**

Difference of Average	Degree of Freedom	t-value	two-sided p-value	t(0.975)
1.452285714	26.83448914	18.95012439	3.97845E-17	2.051830493

Interval estimation for differences of population average:

Degree of reliability	95%
Lower limit	1.295039036
Upper limit	1.609532393

C. Conclusion

From these results, it is apparent that diagnosis of endometriosis is possible by measuring expression levels of HRF gene in menstrual blood. Thus, it is my expert opinion and belief that the claimed invention is enabled for diagnosing endometriosis by measuring expression levels of HRF gene in menstrual blood.

I further declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date: Aug. 12, 2009

Masahiko Kuroda